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A C-Terminal PRES1 Sequence Is Sufficient to Retain Hepatitis B Virus L Protein in 293 Cells

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Analysis of deletion and/or site-specific mutants of the hepatitis B virus (HBV) *env* gene, expressed in human cells, provided clues about the mechanism that retains the L protein, the largest gene product, in a pre-Golgi compartment. Differences in secretability of the analyzed variants suggest that the N-terminal myristic acid and an internal sequence within the PreS1 region function as independent retention signals. N-terminal myristic acid alone neither prevented PreS1 + 2 N-linked glycosylation, which signals cotranslational translocation of the domain, nor strongly inhibited lumenal budding. Thus, myristic acid by itself acts by arresting secretion of lumenal, soluble Env particles. By contrast, the internal retention determinant, mapping in the C-terminal portion of PreS1, also prevented budding. In addition, the presence of this PreS1 segment correlated with the depression of PreS1 + 2 glycosylation. This suggests a connection between L retention and the recently described inhibition of PreS1 + 2 cotranslational translocation. A model can be proposed, according to which HBV surface proteins need to cotranslationally translocate their N-terminal moieties in order to assume a transmembrane topology suitable for particulate assembly and secretion. L protein, whose PreS1 + 2 domain undergoes translocation only posttranslationally, would fail to complete the secretion process. To support this model, we show that forced cotranslational translocation of the PreS1 + 2 domain (by attachment of an N-terminal processed signal sequence) results in secretion of L protein.

INTRODUCTION

A number of enveloped eukaryotic viruses assemble at intracellular membranes, either at the site where their surface proteins first meet the secretory apparatus — i.e., at the endoplasmic reticulum — or in compartments functionally connected to it (Golgi and nuclear envelope). The budding site is thought to be specified by virus-encoded membrane proteins, activating a mechanism that subtracts them from the bulk vesicle flow and allows them to moor in the budding compartment, where interaction with the nucleocapsid takes place (Stephens and Compans, 1988). In the present study, we report on the pre-Golgi retention mechanism exploited by the hepatitis B virus (HBV) L protein, providing evidence that it could be related to the unique topogenetic properties of that glycoprotein.

HBV virion is a 42-nm particle, consisting of a 27-nm icosahedral nucleocapsid surrounded by a lipoprotein envelope, which includes three related glycoproteins (for a review see Blum *et al.*, 1989; Ganem and Varmus, 1987). Virions are assembled at, and bud into, ER or pre-Golgi cisternae. However, an excess of envelope proteins, not incorporated in virions, self-assembles into 22-nm spherical (or, less abundantly, tubular) particles, which are secreted into pre-Golgi cisternae (Patzer *et al.*, 1986; Huovila *et al.*, 1992) and then extracellularly, as hepatitis B surface antigen (HBsAg). HBsAg includes host lipids in a nonbilayer arrangement.

All three envelope proteins are the products of a single HBV open reading frame (ORF *env* or *preS-S*), including three in-phase start codons and a common stop codon (Galibert *et al.*, 1979; Pasek *et al.*, 1979; Valenzuela *et al.*, 1979) (Figs. 1A and 1B). Consequently, the small (S), middle (M), and large (L) envelope proteins share a C-terminal domain (the C-terminal transmembrane domain, CT in this work) and differ in their increasing N-terminal extensions. S coincides with the CT domain (226 aa); M (281 aa) bears a 55-aa domain (preS2 region) N-terminally to the CT domain; L (389 aa) carries a further 108-aa extension (preS1 region) with respect to M, and its amino terminus is myristylated (Persing *et al.*, 1987) (Fig. 1A). S protein is overrepresented in spherical HBsAg, where L is very scarce. This ratio is altered in virions (and HBsAg tubules) where S still prevails but L is more abundant (Heermann *et al.*, 1984). M is present in intermediate amounts in all forms. *In vitro* studies have demonstrated that S alone can drive the HBsAg assembly/secretion process (Ganem, 1991), whereas both S and L are necessary for the assembly of virions (M seems to be dispensable in both phenomena) (Fernholz *et al.*, 1993; Bruss and Ganem, 1991b). When overexpressed in mammalian cells, L strongly inhibits HBsAg secretion (Cheng *et al.*, 1986; Chisari *et al.*, 1986; Persing *et al.*, 1986; Standring *et al.*, 1986; McLachlan *et al.*, 1987; Ou

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and Rutter, 1987). The initial proposal that L inhibitory action is functional to divert the *env* products from HBsAg biogenesis to virion assembly (Persing *et al.,* 1986) was abandoned after the discovery that, in studies using COS-7 cells and SV40 vectors, the inhibition of particulate secretion depends on L N-terminal amino acids (6–13 in the adw subtype, whose PreS1 is 11 aa longer at the N-terminus with respect to other subtypes) and separately on N-terminal myristic acid (Kuroki *et al.,* 1989), while for virogenesis as reconstructed in transfected hepatoblastoma cells, the N-terminus (Bruss and Ganem, 1991b) and actually the N-terminal 5/6 of PreS1 sequence (Bruss and Thommsen, 1994) are dispensable. However, our and other groups have reported on a maintained inhibitory phenotype in L mutants devoid of the myristylated N-terminus, based on experiments using distinct host cell–vector combinations: HepG2 cells and a nonreplicative vector (Prange *et al.,* 1991); HepG2 or 293 cells and a BK virus-based episomal vector (Gallina *et al.,* 1992, 1994); and CV-1 cells and a vaccinia vector (Nemeckova *et al.,* 1994).

The transmembrane topologies of S, M, and L were assumed at first to be similar. The CT domain was thought to traverse at least twice the bilayer thanks to two hydrophobic signals (IS1 and IS2; Fig. 1A), with a hydrophilic region between IS1 and IS2 looping into the cytosol and a second hydrophilic region downstream from IS2 looping into the ER lumen (Eble *et al.,* 1986, 1987; Simon *et al.,* 1988). In M, the PreS2 region is translocated to the lumenal face by downstream signals, consistent with its surface exposition in both HBsAg and virions and with its glycosylation near the N-terminus (Eble *et al.,* 1990). In L, the N-terminal domain (PreS1 + 2 region) was thought to be similarly translocated, owing to the well-established notions that PreS1 carries a receptor binding sequence near the N-terminus (Neurath *et al.,* 1986) and is accessible on virions to proteases and antibodies (Kuroki *et al.,* 1990). Recent evidence, however, suggests that the membrane topology of HBV envelope proteins might be more complex. First, in all three proteins the second hydrophilic region in CT seems to be translocated into the ER lumen only in a fraction of polypeptides, which could explain why CT is only partially glycosylated (Prange and Streeck, 1995). Second, the entire PreS1 + 2 domain in L fails to be cotranslationally translocated and remains at the cytosolic side of the ER membrane (Ostapchuk *et al.,* 1994; Bruss *et al.,* 1994; Prange and Streeck, 1995). Only posttranslationally a fraction of L molecules reorient their N-terminal domain into the lumen (Prange and Streeck, 1995). Heterogeneity in PreS1 + 2 topology is maintained in virions, where some of the PreS1 + 2 domains are hidden (Bruss *et al.,* 1994). The minimal PreS1 sequence supporting HBV virogenesis (the C-terminal 17 aa in adw subtype) overlaps that required for the block of cotranslational translocation in COS-7 cells (Bruss and Thomssen, 1994; Prange and Streeck, 1995). Possibly, then, a dual topology allows L protein to fulfill two crucial functions at different stages of the virus life cycle. A cytosolically located PreS1 + 2 domain might be needed for the interaction of Env proteins with the nucleocapsid components in particle assembly, while a lumenally disposed PreS1 + 2 is the premise to its surface exposition on particles, where it acts as an essential receptor-binding domain.

Here, we describe a study aimed at refining the identification of determinants of L secretion-inhibitory properties. To this purpose, a collection of ORF *env* (ayw subtype) derivatives with mutations focused on the preS1-coding portion was expressed in human 293 cells, and the transport properties of the corresponding products were analyzed. Our results indicate that N-terminal myristic acid and an internal preS1 segment act as independent retention signals, while N-terminal preS1 amino acids are not involved. More interestingly, presence of the internal Pre-Golgi retention signal correlates with the inhibition of PreS1 + 2 glycosylation, an indicator of cotranslational translocation. The observed correlation gives support to a model subordinating HBV surface protein secretion to N-terminal domain cotranslational translocation. Direct evidence for this model is also presented.

MATERIALS AND METHODS

DNA constructions. Plasmid pRPAUG1.2.3ayw (Gallina *et al.,* 1992) carries a 2.4-kb fragment (*Bgl*II, nt 2839–*Bgl*III, nt 1986; positions of nucleotides, nt, in the HBVayw sequence are calculated from an *Eco*RI site as the zero) containing the complete *env* gene and downstream HBV genome through the polyadenylation site (Galibert *et al.,* 1979). HBV sequences are under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) (Manservigi *et al.,* 1990). The vector undergoes episomal replication in human cells as it bears the complete *ori-early* region from BK virus DNA (Milanesi *et al.,* 1984). Plasmid pRPAUG2.3ayw is identical to the already described pRPAUG2.3 (Gallina *et al.,* 1992), except that its HBV insert is from the ayw subtype. Plasmid pRPΔ2-19, formerly cited as pRPpreS1Δ, has been described (Gallina *et al.,* 1994).

The remaining plasmids employed herein were pRPAUG1.2.3ayw derivatives, generated with the help of synthetic oligonucleotides, used as either PCR primers or adapters to modify the original HBV insert. To assemble plasmid pRPAla2, cloned HBV DNA was amplified with the 5′ primer GGAACAAGATCTACAGCATGGCGCAGAAT, which introduces a Gly to Ala mutation at *env* codon 2 (underlined), and the 3′ primer GTTCTCCATGTTCAGCGC (primer 3′-PreS), annealing to HBV nt 165 to 148. The PCR product was digested with *Bam*HI, cutting in the 5′ primer, and with *Xho*I, cutting in the PreS2 coding region (nt 129), and cloned into pRPAUG1.2.3ayw in substitution of the original *Bgl*II 2839–*Xho*I 129 fragment.

Similarly, plasmids pRPΔ2-48, pRPΔ2-69, pRPΔ2-91, and pRPΔ2-99, expressing *env* 5′ deletions, were constructed by PCR with 5′ primers TGACTGAGATCTACCATGGCTGGAGCATTCGGGCTGG,TGACTGAGATCTACCATGGCTCAGGGCATACTACAAAC,TGACTGAGATCTACCATGGCAAGGCAGCCTACCCCGCTG, and TGACTGAGATCTACCATGGCACCTTTGAGAAACACTCATCC, respectively, and with the 3′ primer 3′-PreS. In Δ2-48 and Δ2-69 variants, the 5′ primer was constructed to place the start codon upstream from a naturally occurring Ala codon (underlined). In Δ2-91 and Δ2-99 variants, the primer introduces an extranumerary Ala codon between the AUG and the first nondeleted *env* codon (underlined). In all cases, the initiator AUG is embedded in a strong initiation context (matching the consensus A/GXXAUGG) (Kozak, 1989), which assures that the corresponding deleted protein will be synthesized as the most abundant *env* product. The *Bgl*II–*Xho*I restricted PCR product was reinserted into pRPAUG1.2.3ayw, as above.

The derivatives pRPΔ22-108 and pRPA1a2-Δ22-108 were generated by deleting the indicated *env* codons from pRPAUG1.2.3ayw and pRPAla2, respectively. This was obtained by excising the *Bam*HI 2906–*Eco*RI 3182 fragment from the *env* insert of either plasmid and replacing it with the oligo adapter 5′-GATCCAATGCAGTGGGTTACGTCACCTTAA-5′, thus joining in phase the PreS1 codon 21 to PreS2 AUG (underlined).

For pRPΔ22-123 and pRPAla2-Δ22-123 construction, we took advantage of a previously characterized PCR amplicon of the ayw PreS1 + 2 coding region. That fragment, cloned into a bacterial expression vector, was shown to contain a silent A to G mutation at nt 29, creating a novel *Pst*I/*Bam*HI site, exactly in the position where it is naturally found in the adw subtype (*Valenzuela et al.*, 1979). Fragment *Bam*HI 30–*Xho*I 129 from that clone was substituted for *Bam*HI 2907–*Xho*I 129 into pRPAUG1.2.3ayw and pRPAla2, thus creating an in-frame codon 22–123 deletion in the original *env* insert.

Plasmid pRP-PPL was created by fusing an exogenous signal peptide-coding region 5′ to HBV *env*. For this purpose, a two-stage PCR strategy was adopted. The 5′ primer AGCGTGGCCCCCTTGCCCGGGCAGAATCTTTCCACCAG (a), together with primer 3′ PreS, was used to amplify the PreS coding region, starting from codon 2. Codons 25–30 from the human Preprolactin coding gene (underlined) are appended at the 5′ end in primer (a). In parallel, a double-stranded oligonucleotide covering Preprolactin codons 1–30 (underlined) was generated by PCR extension of the partially hybridizing oligonucleotides CTAGTCAGATCTAACATGAACATCAAAGGATCGCCATGGAAAGGGTCCCTCCTGCTGCTG (b) and GGGCAAGGGGGCCACGCTCTGGCACAGCAGCAGGTTTGACACCAGCAGCAGCAGGAGGGAC (c). Confluence of the first-stage PCR products was obtained by mixing them and running a second PCR round with primers (b) and 3′ PreS. The final fragment, restricted with *Bgl*II and *Xho*I, was substituted into pRPAUG1.2.3ayw.

All of the above-described PCR reactions were performed with the high fidelity *Pfu* polymerase (Stratagene). The sequence of plasmids produced by the use of PCR products or oligo adapters was confirmed by dideoxynucleotide sequencing. Plasmids were purified for transfections with Qiagen.

Cells and transfections. Cells 293 (Graham *et al.*, 1977) were mantained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS). For transfections, 3×10^6 cells were plated in 25-cm^2 flasks. After 1 day, the complete medium was changed and cells were incubated for 4 hr at 37° and 5% CO_2. Plasmid DNA (12.5 μg) was then administered according to the calcium phosphate method (Gorman, 1985) and left overnight. The cell monolayer was washed once with Hebs solution (Gorman, 1985), fresh complete medium was added, and cells were incubated for another 24 hr. For metabolic labeling, cells were washed twice in methionine-free DMEM without FBS and incubated in the same medium for 45 min. Then, starvation medium was discarded and 2 ml prewarmed Met-free DMEM with 15 μl/ml Pro-mix (Amersham; 150 μCi of [^{35}S]methionine, ~1 × 10^3 Ci/mmol) was added. After 3 hr, cells were either harvested for subsequent immunoprecipitation or chased for a further 18 hr in 5 ml complete medium. This was followed by immunoprecipitation of Env proteins from cell lysates and from the culture medium. To reveal N-terminal myristylation in Env proteins, cells were labeled overnight with 1 mCi/flask of [^{3}H]myristic acid (Amersham; ~50 Ci/mmol) in DMEM, 1% FBS.

Immunoprecipitations. After either pulse or pulse-chase labeling, transfected cells were washed once with PBS and dissolved in 750 μl ice-cold lysis buffer [10 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 1 m*M* phenylmethyl sulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin]. Cell lysates were spun for 5 min in a Eppendorf minifuge (4°), supernatants were transferred to fresh tubes, and 750 μl of RIPA buffer (0.1% SDS in lysis buffer) was added. After another centrifugation, supernatants were transferred again and 8 μl of a PBS suspension of Sepharose beads conjugated to anti-HBsAg sheep immunoglobulins (~1 μg/μl Sepharose slurry) were added. Samples were shaken end-over-end at 4° for 3 hr. In pulse-chase experiments, culture media were clarified by centrifugation and immunoprecipitated with 8 μl Sepharose-anti-HBsAg conjugate. Sepharose beads were then washed extensively in 1:1 lysis buffer–RIPA buffer and twice in distilled water. Unless immunocomplexes had to be exposed to enzymatic treatment, they were directly denatured by boiling (3 min) in 25 μl SDS–PAGE loading buffer. After electrophoresis (12% resolving acrylamide) gels were fixed in 25% methanol, 10%

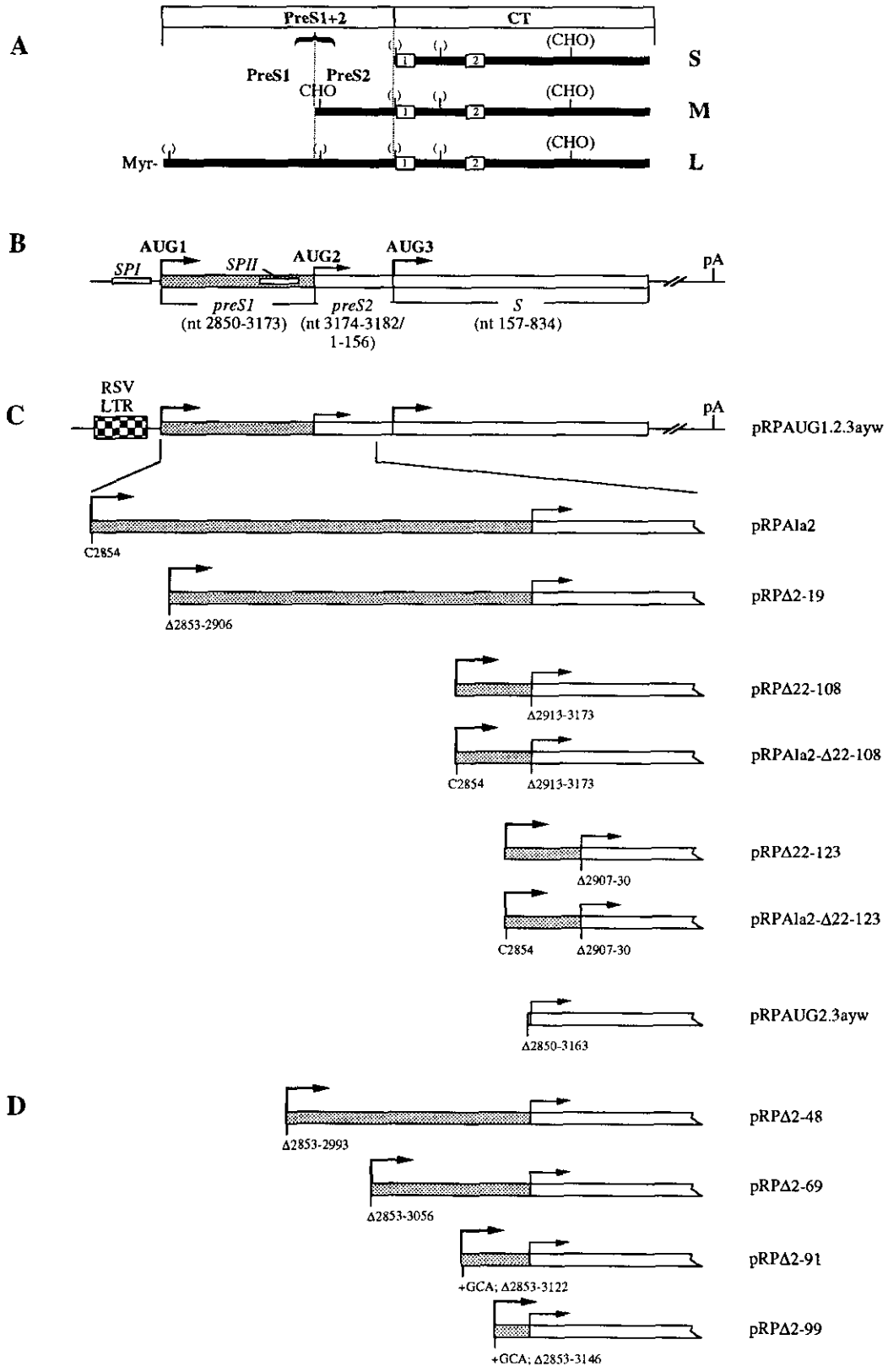

FIG. 1. (A) Domain organization of the HBV envelope proteins S, M, and L. N-linked glycosylation sites within PreS1, PreS2, and CT (C-terminal transmembrane) domain are represented as vertical bars. (CHO), glycosylation site exploited in a fraction of polypeptides; CHO, fully exploited site; (), normally unexploited site. Myr, myristic acid adduct modifying L protein Gly2. Boxes 1 and 2 within the CT domain represent IS1 and IS2, respectively. (B) Structure of the *env* ORF on the HBV genome. *SPI* and *SPII* boxes indicate the promoters driving the transcription of the entire gene, or of the PreS2-S coding region, respectively. ATG start codons are shown; the strong initiators ATG1 and ATG3 are represented by thick,

TABLE 1

Behavior of *env* Products with Respect to Secretion, Co/Posttranslational Modifications, and Budding Competence

Plasmid	Mutations in the full-length protein		Secretion[a]	Covalent modifications				Lumenal HBsAg[d]
				Myristylation[b]	Glycosylation[c]			
	Point m./fusions	Deletions			PreS1	PreS2	s	
pRPAUG2.3ayw	/	Δ1–108	+++	/	/	p/m+	p/m+	50%
pRPAUG1.2.3ayw	/	/	/	p/m+	p/nu	p/nu	p/m+	1%
pRPAla2	Gly to Ala2	/	/	/	p/m±	p/m±	p/m+	2%
pRPΔ2-19	/	Δ2–19	/	/	/	p/m±	p/m+	NT
pRPΔ22-108	/	Δ22–108	/	p/m+	p/m+	p/m+	p/m+	39%
pRPAla2-Δ22-108	Gly to Ala2	Δ22–108	++	/	p/m+	p/m+	p/m+	NT
pRPΔ22-123	/	Δ22–123	/	p/m+	p/m+	/	p/m+	NT
pRPAla2-Δ22-123	Gly to Ala2	Δ22–123	++	/	p/m+	/	p/m+	NT
pRPΔ2-48	/	Δ2–48	/	/	/	p/m±	p/m+	NT
pRPΔ2-69	/	Δ2–69	/	/	/	p/m±	p/m+	4%
pRPΔ2-91	extra Ala2[e]	Δ2–91	+	/	/	p/m+	p/m+	NT
pRPΔ2-99	*extra Ala2*[e]	Δ2–99	++	/	/	p/m+	p/m+	NT
pRP-PPL	extra aa 1–30 from h-Preprolactin[f]	/	++	/	p/m+	p/m+	p/m+	NT

[a] Secretion as checked by visual evaluation of immunoprecipitation autoradiographies.
[b] N-terminal myristilation at PreS1 Gly2, as assayed by [^{3}H]myristic acid labeling of transfectants and immunoprecipitation.
[c] N-linked glycosylation: p/m+, present and modified; p/m±, present and scarcely used; p/nu, present but not used.
[d] Determined after Prange *et al.*, 1991. Extracted Env was assayed with Auszyme (Abbott); NT, not tested.
[e] The extranumerary amino acid is between Met1 and the first not deleted PreS1 residue.
[f] Preprolactin signal sequence was fused N-terminally to PreS1 Gly2.

acetic acid (30 min), enhanced in Amplify solution (Amersham), dried, and exposed to X-ray film.

Endoglycosidase H and protease digestions. For endoglycosidase H digestion, ^{35}S-labeled Env immunocomplexes bound to Sepharose beads were drained of water and resuspended in 25 μl of 50 m*M* sodium citrate, pH 5.5, 0.1% SDS. After 1 min boiling, 3 U recombinant endoglycosidase H (Boehringer) was added, and the reaction mixtures were incubated at 37° overnight, under a drop of mineral oil. Mock reactions were run in parallel without added enzyme. Reactions were then adjusted to 1× SDS–PAGE loading buffer and used for electrophoresis and autoradiography. For trypsin digestion, immunoprecipitated Env proteins were resuspended in 25 μl 20 m*M* Tris–HCl, pH 8, 1 m*M* $CaCl_2$, 0.5 μg/ml trypsin (Boehringer; absent in mock reactions) and incubated at room temperature for 15 min. The reaction was stopped by adding 0.5 μl 100 m*M* PMSF and incubating on ice for 15 min. Samples were then denatured in a final 1× loading buffer and processed for autoradiography as above.

Subcellular fractionations. Membrane-associated as opposed to extractable (lumenal) intracellular Env protein was analyzed according to the freeze–thaw protocol of Streeck and co-workers (Prange *et al.*, 1991). Transfected cells were processed without radioactive labeling. After separation of the soluble extract, membrane-associated proteins were solubilized in 0.2% NP-40 in PBS; soluble extracts were adjusted to the same NP-40 concentration. Both fractions were diluted 1:10 in DMEM–10% FBS, and tested for Env protein content with a commercial enzyme immunoassay kit (Auszyme, Abbott) according to the instructions of the manufacturer.

Sucrose gradients. The particulate nature of secreted Env variants was analyzed by rate-zonal sedimentation. An aliquot (500 μl) of transfected cell medium was layered onto 11.5 ml 5–30% sucrose gradients in 10 m*M* Tris–HCl, pH 7.5, 130 m*M* NaCl, 1 m*M* EDTA (TEN buffer) formed in SW40 rotor (Beckman) tubes. Gradients were centrifuged for 16 hr at 24 krpm (4°), and fractionated in 24 ~0.5-ml fractions. Each fraction was diluted 1:3 in TEN and assayed with the Auszyme kit. Peak fractions were resolved in SDS–PAGE and transferred to nitrocel-

tall, angled arrows; the weak initiator ATG2 is represented by a short angled arrow; pA, HBV polyadenylation site. (C, D) *Env* variants utilized in this work. The expression region of the ancestor construct pRPAUG1.2.3ayw is shown entirely, while only the enlargement of the *preS1–preS2* region carrying deletion and/or point mutations is depicted for the remaining constructs. RSV LTR, Rous sarcoma virus long terminal repeat. ATG symbols are as in B. Representation of the *SPII* element was omitted. The numbers under the gene box refer to *preS1–preS2* mutations, e.g., C2854, G to C mutation at position 2854; Δ2853-2906, deletion of nucleotides 2853–2906. +GCA, insertion of an Ala codon between the ATG codon and the first nondeleted *preS1* codon.

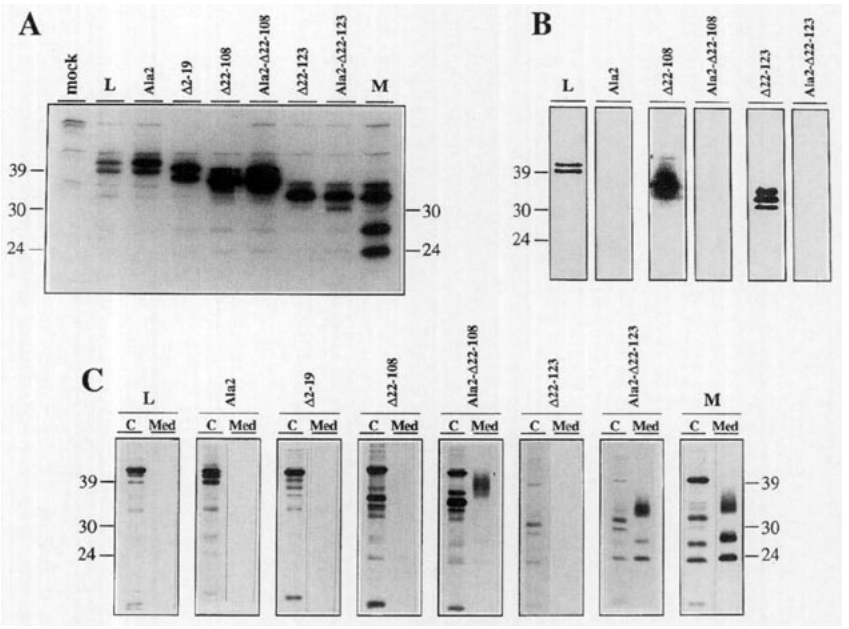

FIG. 2. Synthesis and secretion of Env protein variants. Transfected cells expressing the indicated Env variant were pulse-labeled with [^{35}S]-methionine and lysed for immunoprecipitation with anti-HBsAg antibodies. Immunoprecipitated proteins were resolved by SDS–PAGE and visualized by autoradiography (A). To check N-terminal myristylation, transfectants were labeled overnight with [^{3}H]myristic acid and similarly processed (B). For studying secretion, pulse-labeled cells were chased overnight in complete medium, and comparable amounts of cellular lysates (C) and culture supernatants (Med) were immunoprecipitated and analyzed by SDS–PAGE (C). Position and molecular mass (in kDa) of intracellular, unglycosylated wt L (39), M (30), and S (24) is indicated. In this figure as well as in Figs. 3–5, a band of apparent molecular weight slightly higher than glycosylated L recurs as a major immunoprecipitation contaminant.

lulose for Western blot analysis. Using a goat anti-HBsAg (Chemicon) as the primary antibody, Env proteins were revealed following the ECL chemiluminescence protocol (Amersham). Under Western blot conditions, the goat antiserum was reactive mainly to PreS1 + 2 epitopes.

RESULTS

PreS1 carries two independent retention determinants. All of the *env* mutants (Figs. 1C and 1D) were inserted into the expression site of the episomal plasmid pRPRSV, under the transcriptional control of RSV LTR (Manservigi *et al.*, 1990). In these constructs, the activity of the autologous HBV SPII promoter (Schaller and Fischer, 1991) (Fig. 1B), when not deleted, is very weak (Gallina *et al.*, 1992, 1994). Moreover, the 5′-most *env* AUG in LTR-driven transcripts is in a strong initiation context. As the result, the predominantly expressed protein is the full-length *env* mutant product, with minor M + S or S amounts expressed owing to ribosome leaky scanning and to residual SPII activity. The same expression pattern is observed with a control construct expressing wt L (pRPAUG1.2.3ayw; Fig. 1C), whereas an M-expressing control (pRPAUG2.3ayw; Fig. 1C) generates roughly equimolar amounts of S, due to high rate read-through at the M start codon (Gallina *et al.*, 1992). Overexpression of the full-length *env* product relative to M and/or S distinguishes the BK-based expression system used in the present study from the SV40-based vector/COS-7 cells system.

A first panel of L variants (Fig. 1C; Table 1) composes a factorial design, allowing us to evaluate the following factors as possibly involved in L protein retention: (i) N-terminal myristic acid (present or removed by Gly-2 to Ala-2 mutation); (ii) PreS1 N-terminus (amino acids 1–19 present or removed by deletion); (iii) PreS1 amino acids 22–108 (present or removed by deletion). Two variants (Δ22-123 and Ala2-Δ22-123) in which the internal deletion extends to PreS2 aa 1–13 were also included.

In preliminary transfection experiments, the presence of *env* products in 293 cells was revealed by pulse-labeling with [^{35}S]methionine, immunoprecipitation from cell lysates with anti-HBsAg antibodies, gel electrophoresis, and autoradiography. The resulting electrophoretic patterns (Fig. 2A) are complex because of M and S coexpression and because of the presence of glycosylated forms for each expressed polypeptide. In all lanes, S accounted for the two lowest bands, representing its unglycosylated (24 kDa) and monoglycosylated forms. M was synthesized in three forms: non- (30 kDa), mono-, and diglycosylated. It was observed as the largest product in pRPAUG2.3ayw transfectants and as a minor product of constructs including the complete PreS2 coding region. L (pRPAUG1.2.3ayw transfectants) is represented by two bands: non- (39 kDa) and monoglycosylated. For mutant proteins, Ala 2 was indistinguishable from L. Mutant Δ2-19 exhibited an intense doublet, plus a slower migrating, very faint band. Mutants Δ22-108 and Ala2-Δ22-108 were represented by four bands and mutants

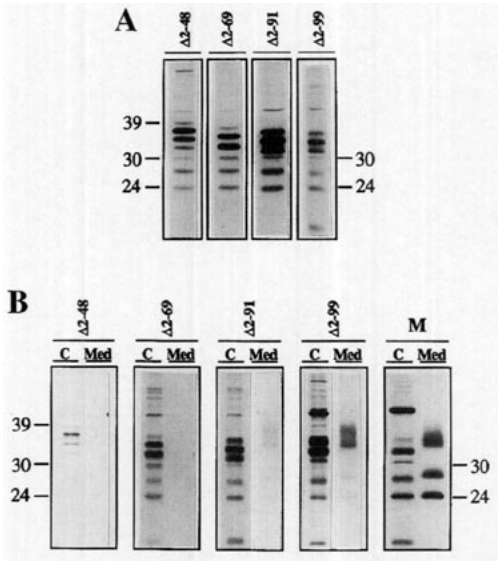

FIG. 3. Mapping of PreS1 intracellular retention determinant. The indicated transfectants were pulse-labeled (A) or pulsed-chased (B) and subsequently processed as described in the legend to Fig. 2. Position and molecular mass of wt S, M, and L (unglycosylated) is shown.

Δ22-123 and Ala2-Δ22-123 by three bands. Mutant glycosylation is further discussed below. A band of variable intensity, migrating slightly slower than glycosylated L, was observed in most experiments (Figs. 2–4); it represents a contaminant from immunoprecipitation (it is seen also in mock-transfectant immunoprecipitation, Fig. 2A). The above interpretation of electrophoretic patterns was further confirmed by Western blot experiments with an anti-PreS2 monoclonal antibody (data not shown).

The presence/absence of the myristic acid adduct in Env variants conserving the modifiable N-terminus and in their Ala2 counterparts was verified by labeling transfectants with [^{3}H]myristic acid, followed by immunoprecipitation and gel electrophoresis. ^{3}H-labeled bands matching the ^{35}S-labeled bands of the same proteins were observed for L, Δ22-108, and Δ22-123, but not for their Ala2 derivatives, as expected (Fig. 2B, Table 1).

To study *env* products exported after transfection and a 3-hr [^{35}S]methionine pulse/18 hr chase, proteins were immunoprecipitated from cell lysates and, in parallel, from culture medium (Fig. 2C) and separated by gel electrophoresis. Comparison of patterns of intracellular proteins in pulse (Fig. 2A) and in pulse-chase (Fig. 2C, lanes C) experiments indicated that, during chase time, Env proteins underwent only mild intracellular degradation (signaled by bands migrating faster than S polypeptides). Analysis of extracellular proteins (Fig. 2C, lanes Med) showed that mutant variants could be divided into two classes (Table 1). Those including PreS1 aa 22–108 and/or the N-terminal myristyl adduct (Ala2, Δ2-19, Δ22-108, and Δ22-123) were not exported, in analogy to wt L. On the contrary, variants lacking both elements (Ala2-Δ22-108, Ala2-Δ22-123) were secreted, although with a slightly reduced efficiency compared to wt M. For extracellular forms, evidence that they were genuinely secreted, and not released into the medium after cell lysis, was given by the increase in apparent molecular weight relative to intracellular counterparts, due to Golgi trimming of oligosaccharides (Fig. 2C). Coherently, they were found to be resistant to endoglycosidase H (data not shown).

This preliminary assessment showed that the PreS1 C-terminal 4/5th was sufficient to determine ER retention. N-terminal myristic acid also caused retention, while the N-terminal portion of PreS1 appeared to be devoid of retention signals distinct from the lipid adduct.

Restriction of the internal retention signal to PreS1 stretch 71–99. In order to map the internal PreS1 retention determinant more precisely, four additional mutants were created, progressively extending the deletion toward the PreS1 C-terminus. Variants Δ2-48, Δ2-69, Δ2-91, and Δ2-99 (Fig. 1D; Table 1) were analyzed in pulse experiments to verify their expression. All of them exhibited a triplet in SDS–PAGE, with the highest form either in traces (Δ2-48 and Δ2-69) or intense (Δ2-91 and Δ2-99; see below) (Fig. 3A). As usual, minor amounts of M forms (partially overlapping the full-length product) and S forms were coexpressed.

Mutants were then tested in pulse-chase experiments for secretion. It was apparent that while mutants Δ2-48 and Δ2-69 were not secreted, Δ2-91 was appreciably secreted and Δ2-99 displayed an overt secretory phenotype (Fig. 3B; Table 1).

The PreS1 stretch essential for retention was thus restricted by these results to amino acids 70–99. Altogether, data from point and deletion mutants analysis indicate that a short segment at the C-terminus of PreS1

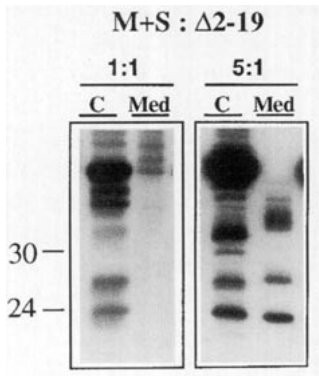

FIG. 4. Co-oligomerization of mutant Δ2-19 with wt S and M. Construct pAFΔ2-19 and pAFSSlink were cotransfected into 293 cells. The relative amounts of plasmids were adjusted to obtain either equimolar Δ2-19 and M + S synthesis (lanes 1:1) or a large excess (~fivefold) of M + S relative to Δ2-19 (lanes 1:5), while keeping the total DNA amount (12.5 μg/25-cm^2 flask) fixed. Transfectants were pulse-chased and processed as described in the legend to Fig. 2. Position and molecular mass of wt S and M is shown.

can determine *per se* a deep depression of L secretion as HBsAg.

The above inferences assume that deleted L variants were not largely misfolded and that all of the retained variants were not arrested in transport by ER quality control mechanisms (Rose and Doms, 1988). A direct argument in favor of this assumption is the comparable stability of the considered variants throughout the time of the experiments. Furthermore, several observations suggested that the shared CT domain was not grossly misfolded in all of the analyzed proteins. In fact: (i) All of the tested variants were recognized both in ELISA and in immunoprecipitation by conformation-dependent monoclonal antibodies, whose reactivity needs a well-folded S domain (data not shown). (ii) Mutants were found to covalently dimerize intracellularly, via disulfide bridges, like wt Env polypeptide (data not shown). (iii) When the retained Δ2-19 variant was coexpressed with roughly equimolar amounts of M + S polypeptides, the secretion of S and M was substantially reduced (Fig. 4). Vice versa, a large excess of M + S to Δ2-19 (which simulates the molar ratios observed in earlier work based on the COS-7–SV40 vector system) promoted the secretion of all Env forms (Fig. 4). This suggests that the Δ2-19 CT domain can co-oligomerize with wt S and M, i.e., that it is seemingly well-folded; large amounts of coassembled M + S polypeptides suppress the Δ2-19 secretion-inhibitory phenotype.

An altered folding of the PreS1 + 2 domain after the creation of large deletions, on the other hand, is not only possible, but expected. However, the observed secretion of deletion variants lacking PreS1 + 2 extensions clearly shows that not all lesions of PreS1 + 2 generate an unexportable polypeptide.

Strong retention correlates with the blockade of PreS1 + 2 glycosylation. The removal of portions of the L PreS1 + 2 domain has been previously shown to lead to *de novo* glycosylation at the normally silent PreS1 + 2 sites (Asn 4 within PreS1 and/or Asn 112 at the PreS2 N-terminus; Fig. 1A), irrespective of the conservation or abolition of N-terminal myristylation (Gallina and Milanesi, 1993; Bruss and Thomssen, 1994; Prange and Streeck, 1995). Consistent with the notion that N-linked glycosylation takes place cotranslationally [N-oligosaccharyl transferase being a component of the ER translocation pore and attaching the sugar core on the nascent polypeptide, while it emerges into the ER lumen (Kelleher *et al.*, 1992)], the lack of PreS1 + 2 glycosylation in wt L has been recently explained with the block of its cotranslational translocation, mediated by a sequence located at the C-terminus of PreS1 (Bruss and Thomssen, 1994; Prange and Streeck, 1995). Glycosylation of deleted mutants would descend from the ablation of that sequence. The glycosylation phenotype of L variants in our work confirms and extends this concept, suggesting that the determinant of PreS1 + 2 cytosolic anchorage and the L internal retention determinant overlap.

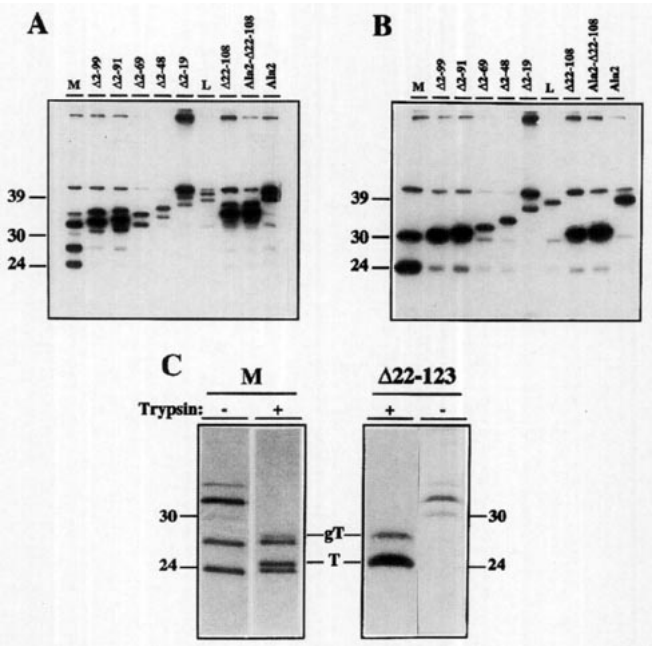

FIG. 5. N-linked glycosylation of Env variants. Immunoprecipitated proteins from pulse-labeled transfectants were mock-treated (A) or treated (B) with endoglycosidase H in 50 m*M* sodium citrate, 0.1% SDS, before SDS–PAGE analysis. Position and molecular mass of unglycosylated S, M, and L is shown. (C) As an alternative, proteins were digested (+) or mock-digested (−) with trypsin in 20 m*M* Tris–HCl, pH 8, 1 m*M* $CaCl_2$ and electrophoresed. Position and molecular weight of undigested, unglycosylated M is indicated, along with that of the glycosylated and unglycosylated Env largest proteolytic fragment (gT and T, respectively).

Closer scrutiny of the electrophoretic pattern of L mutants including either or both PreS1 + 2 glycosylation sites (Table 1, Figs. 2A and 3A), defines in fact two classes: mutants exhibiting an intense diglycosylated (deletions Δ22-123, Ala2-Δ22-123, Δ2-91 and Δ2-99) or triglycosylated (Δ22-108 and Ala22-Δ22-108) form and mutants which, similar to L, are devoid of a second glycosylation (mutant Ala2) or show only a faint trace of it (deletions Δ2-19, Δ2-48 and Δ2-69) (Table 1). To confirm that the slower migrating forms correspond to distinct glycoisomers, mutants were treated with endoglycosidase H. As expected, in all cases the higher bands were shifted to the fastest migrating form (Fig. 5A and 5B). Furthermore, as the CT domain in turn includes two potential, though typically silent, N-glycosylation sites (Asn 3 and 59, counting from the CT N-terminus) in addition to the normally used Asn 146 (Fig. 1A), we looked for a formal proof that the newly modified sites in di- and triglycosylated mutants were indeed the PreS1 + 2 sites. To this purpose, di/triglycosylated variants, after immunoprecipitation, were exposed to trypsin, which selectively destroys the PreS1 + 2 domain through Arg 156. Proteolysis constantly shifted all variants to the molecular

weight expected for a protein extending from Ile157 through the CT domain C-terminus and partially glycosylated at the CT site (shown for Δ22-123, Fig. 5C). The disappearance of di- and triglycosylated forms proves that the additional oligosaccharides are actually attached at PreS1 + 2 sites.

Taking the novel PreS1 + 2 glycosylation as evidence that the blockade of cotranslational translocation has been relieved, the above data suggest that the core determinant of blockade can be mapped at the PreS1 C-terminus, in agreement with earlier suggestions (Bruss and Thomssen, 1994; Prange and Streeck, 1995). More precisely, our data restrict such a sequence to the stretch 70–91, as mutant Δ2-69 showed a strongly depressed PreS2 glycosylation, while Δ2-91 was efficiently modified at the same site. In our experiments, the cytosolic anchorage (lack of glycosylation) of PreS1 + 2 was not complete unless the entire PreS1 domain was included (and irrespective of the presence of N-terminal myristic acid). An apparent correlation between PreS1-induced retention of a mutant and failure to efficiently glycosylate its N-terminal domain emerges from our data, as indicated by the colocalization of the core determinant of both phenomena. This correlation is in agreement with the observation that extracellular Env polypeptides are regularly PreS-glycosylated (see Figs. 2C and 3B). By contrast, in mutants in which retention seemed to descend exclusively from the N-terminal myristic acid and the internal retention element was eliminated (Δ22-108 and Δ22-123), the domain was efficiently glycosylated (cotranslationally translocated).

Strong impairment of HBsAg lumenal budding in variants including the PreS1 retention determinant. The conclusion that myristic acid and a C-terminal PreS1 element act separately in causing L intracellular retention and that the latter, but not the former, correlates with inhibition of PreS1 + 2 glycosylation prompts the question of whether the two retention determinants interfere in the same way with HBsAg biogenesis. It has been previously shown (Prange *et al.*, 1991) that a mutant S protein, bearing an N-terminal myristic acid adduct which blocks its secretion, is nonetheless able to bud as lumenal HBsAg and as such can be detected in the soluble extract in a cell fractionation protocol. We applied the same analysis to a choice of Env variants: M, L, Ala2, Δ2-69, and Δ22-108. Cell transfectants producing each variant were subjected to a double freeze–thaw treatment, followed by a low speed centrifugation to eliminate nuclei and cytoskeleton and by an ultracentrifugation step to separate a soluble fraction (the pool of cytosol plus the content of intracellular cysternae) from cell membranes. Soluble and membrane fraction were then tested with an anti-HBsAg EIA. It turned out (Table 1) that while M and, to a lower extent, Δ22-108 were detected in the soluble extract (50 and 39% of total intracellular HBsAg, respectively), L, Ala2, and Δ2-69 were more strictly membrane-associated (1, 2, and 4% in the soluble extract, respectively). In addition, when the extractable fraction of M and Δ20-108 was resolved in a cesium chloride gradient, both proteins banded at the bouyant density (~1.2 g/ml) expected for the HBsAg lipoprotein particle (data not shown). Taking into account that the extraction procedure is inefficient, this result supports the interpretation that variants, including the internal retention determinant (wt L, Ala2, and Δ2-69), were arrested at a transport stage preceding HBsAg budding. By contrast, a mutant (Δ22-108) whose retention is attributable to myristylation was not deeply depressed in budding. Thus, it appears that, in contrast to the internal PreS1 element, the myristic acid *per se* did not strongly inhibit HBsAg biogenesis, at least in the mutant analyzed here. The myristic-induced retention is possibly a HBsAg lumenal anchoring event distinct from that blocking L protein transport.

An L chimera with PreS1 + 2 cotranslational translocation forced by an exogenous signal sequence is secreted. The observed correlation between retention mediated by the PreS1 element and inhibition of PreS1 + 2 glycosylation might indicate a connection between retention and inhibition of PreS1 + 2 cotranslational translocation. Under this hypothesis, results exposed so far can be accommodated in a model in which HBV Env proteins have the absolute need to cotranslationally translocate their N-terminal domain into the ER lumen in order to assume a transmembrane folding competent to complete the HBsAg assembly-budding process. Failure to cotranslationally translocate the N-terminal domain — in natural L and in a number of L variants — would lead to a folding inhibitory of HBsAg secretion.

An immediate prediction of this model is that forcing PreS1 + 2 cotranslational translocation, by addition of an N-terminal signal sequence, should generate a secreted L, in spite of the presence of a complete PreS1 + 2 region. To test this issue, we fused a heterologous signal peptide (amino acids 1–30 from human Preprolactin) to L protein Gly2 (Fig. 6A). This mutant (PPL) was expected to be transported to ER secretory pores at the stage of nascent polypeptide and to have PreS1 + 2 immediately translocated into ER lumen through IS1. The fusion abolished N-terminal myristylation and was expected to leave seven Preprolactin extra amino acids at the N-terminus after processing of the signal sequence. (Fig. 6A). When synthesized in 293 cells, PPL gave rise in pulse experiments to four closely migrating bands (Fig. 6B). After treatment with endoglycosidase H, bands were shifted to the lowest one, demonstrating their nature of mono-, di-, and triglycosylated forms (Fig. 6D). The nonglycosylated PPL displayed an only slightly reduced mobility relative to unmodified L, consistent with quantitative processing of the signal sequence. Moreover, PPL additional sugars map at PreS1 + 2 sites, as di- and triglycosylated bands disappeared after tryptic digestion of the PreS1 + 2 domain (data not shown). (A chimera similar

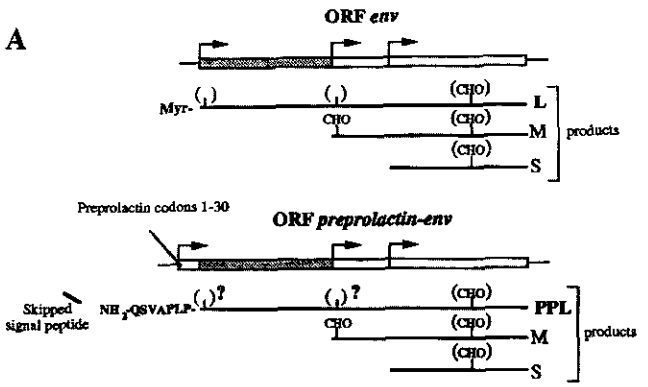

FIG. 6. (A) Comparison of the structures of the natural *env* gene, of the *preprolactin–env* fusion gene, and of the expected products. Symbols are as in Fig. 1. Question marks near the normally unused PreS1 + 2 glycosylation sites signal the uncertainty *a priori* as to their usage in the PPL chimera; representation of unused CT glycosylation sites and of IS1-2 was omitted. (B–D) Glycosylation and secretion of PPL, as studied by immunoprecipitation. Cell transfectants synthesizing the indicated proteins were either pulse-labeled and immediately processed for immunoprecipitation (B) or chased overnight after pulse; in the latter case, cell extracts and medium were analyzed in parallel (C). (D) The immunoprecipitated proteins from pulse-labeled cells were treated (+) or mock-treated (−) with endoglycosidase H (E.H.). The position and the apparent molecular weight of PPL and wt L is shown.

to PPL, with multiple Arg/Lys to Gln mutations in PreS1 + 2, has been expressed in rabbit reticulocyte lysate-canine microsomes. In that system, the chimera was shown to be uncompletely freed of signal peptide and to be glycosylated once at either PreS1 + 2 site; Ostapchuk *et al.*, 1994).

Most importantly, in pulse-chase experiments PPL was abundantly immunoprecipitated from the culture medium along with coexpressed M and S (Fig. 6C) and exhibited the typical Golgi-glycosylated phenotype. Accordingly, extracellular PPL sugars were resistant to endoglycosidase H (Fig. 6D). That PPL was secreted as HBsAg was demonstrated by its sedimentation in sucrose gradients. As shown in Fig. 7, the Env proteins secreted by pRP-PPL cell transfectants behaved as high molecular weight particles, comigrating in sucrose gradients with control M + S particles secreted by pRPAUG2.3ayw transfectants.

Thus, early sequestration of the PreS1 + 2 domain at ER translocation channels eludes the events that would otherwise inhibit HBsAg biogenesis; the complete PreS1 + 2, forced into ER lumen from the N-terminus, loses its inhibitory properties.

DISCUSSION

HBV Env proteins are peculiar in their ability to escape the condition of membrane proteins, by a unique process of assembly/budding in the absence of Env-nucleocapsid interactions. Strictly speaking, all three Env variants are pre-Golgi retained proteins. Indeed, they never reach the Golgi apparatus as transmembrane molecules, as indicated by the absence of Golgi-modified oligosaccharide moieties in intracellular Env forms. Mutagenesis studies

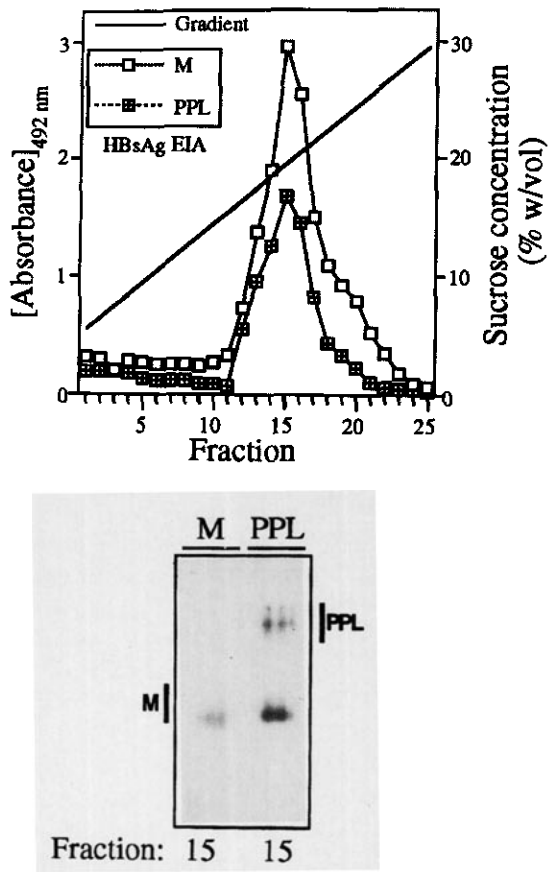

FIG. 7. Assembly of secreted PPL and wt M proteins into lipoprotein particles. Media from transfectants secreting the indicated protein were harvested 48 hr posttransfection. Equal amounts of clarified medium from either transfection was layered on the top of a preformed 5–30% sucrose gradient and centrifuged (Beckman SW40 rotor, 35 krpm, 16 hr 4°). Gradient fractions were assayed for HBsAg (top) by a an enzyme immunoassay (EIA; Auszyme, Abbott). Peak fractions were further analyzed by Western blot (bottom), using a goat anti-HBsAg serum which reacts to PreS1 + 2 nonconformational epitopes. The position of M and PPL is indicated by vertical bars. The low PPL signal in the EIA assay compared to the amount detected by Western blot suggests that exposed PreS1 + 2 in PPL partially masks the CT *a* determinant recognized by the assay.

on the CT domain failed to generate retention-minus mutants, i.e., transmembrane polypeptides with the acquired ability to travel to plasma membrane (Bruss and Ganem, 1991a; Prange *et al.*, 1992). Thus, the retention mechanism is better fitted by an aggregation model, describing retention as the effect of Env oligomerization and exclusion from membrane domains involved in vesicle release, than by a model relying on localized retention signals (Rothman and Orci, 1992; Pelham and Munro, 1993). However, the M and S variants are able to bud into pre-Golgi cisternae in the form of HBsAg particles, which quickly traverse the Golgi with the soluble cargo and are secreted. In contrast, L is inhibited in HBsAg biogenesis and transinhibits M and S secretion.

Here, we present evidence that the C-terminal portion of the L PreS1 region is involved in HBsAg biogenesis inhibition, as studied in a 293 cell–BK virus vector expression system, and that the inhibitory effect might be a consequence of the special PreS1 + 2 translocation mechanims. First, we show that N-terminal myristic acid and the C-terminal 4/5 of PreS1 act as separable determinants of HBsAg secretion inhibition. We then restrict the PreS1 C-terminal determinant to the sequence 70–99, which overlaps the PreS1 stretch implicated in the block of PreS1 + 2 domain cotranslational translocation. Also, it partially overlaps the minimal PreS1 extension (aa 103–119, adw) needed for the virus assembly-proficiency of an L deletion mutant (Bruss and Thomssen, 1994). Furthermore, the observation that a mutant whose transport block is caused only by N-terminal myristylation is not strongly inhibited in HBsAg budding, while nonmyristylated mutants including the PreS1 C-terminal element, like wt L, are blocked as transmembrane molecules, suggests that the PreS1 C-terminal element might act dominantly in the natural molecule, with myristic acid playing an accessory role or even none, spuriously anchoring HBsAg particles after lumenal budding in mutants internally deleted of the PreS1 determinant.

Since N-linked glycosylation is a well-established indicator of cotranslational translocation, the correlation between a depressed or absent PreS1 + 2 N-glycosylation and the inhibition of HBsAg budding attributable to PreS1 C-terminus raises the possibility that the mechanisms involved in blocking cotranslational translocation (Ostapchuk *et al.*, 1994; Bruss *et al.*, 1994; Prange and Streeck, 1995) and that blocking HBsAg biogenesis are strictly related. To support this hypothesis, we show that a forced cotranslational translocation of the L PreS1 + 2 domain, induced by an exogenous signal sequence, renders L secretable via HBsAg budding, in spite of the presence of the entire N-terminal domain.

We tentatively propose to collect the above observations in a model, which is an adaptation of those already suggested for L protein topogenesis (Ostapchuk *et al.*, 1994; Bruss and Thomssen, 1994; Bruss *et al.*, 1994; Prange and Streeck, 1995). This revised model assumes that Env protein N-terminal extension determines not only its own mode of translocation and the overall membrane disposition of the polypeptide, but also the intracellular fate of the protein. S and M proteins bear a 3- and 58-aa hydrophilic N-terminal extension, respectively, which can be cotranslationally translocated into ER lumen in a backward fashion by the proximal CT signal IS1. L protein extension (166 aa), on the other hand, includes an element (PreS1 aa 70–99) which inhibits PreS1 + 2 cotranslational translocation and imposes initially a distinct membrane topology –PreS1 + 2 and possibly IS1 cytosolically disposed. According to the model being proposed, the final PreS1 + 2 and/or CT domain folding in L would remain incompatible with HBsAg budding, even if posttranslationally a fraction of PreS1 + 2 domains gains a lumenal localization thanks to an as yet uncharacterized mechanism (Ostapchuk *et al.*, 1994; Bruss *et al.*, 1994; Prange and Streeck, 1995).

Not all facts are in agreement with the proposed model. A major discrepancy is with the aforementioned data from the COS–SV40 expression system. Kuroki *et al.* (1989) and Bruss and Thomssen (1994) have shown that (i) all adw L derivatives lacking the myristylated N-terminus are always appreciably secreted in COS cells, although secretion is stronger in variants deleting most of the PreS1 region; (ii) PreS1 + 2 N-glycosylation is absent until PreS1 is deleted to adw aa 110, i.e., until only the C-terminal 9 aa of PreS1 are left. Thus, in the COS–SV40 system the block of cotranslational translocation and that of HBsAg secretion appear uncoupled, and the block of cotranslational translocation seems to be an all-or-none event, with the critical PreS1 residues confined in the adw stretch 102–110, corresponding to ayw aa 91–99. A possible explanation of differences between the aforementioned data and those reported here could be in the cell milieu and/or in a vector effect. However, when a choice of L deletion mutants was expressed in either COS-7 or HepG2 cells via the BKV-based constructs, Env glycosylation patterns and secretion phenotypes reproduced the situation in 293 cells (E.G., A.G., G.M., unpublished results). The same held true when key mutants were transferred into a SV40-based vector (which did not drive the synthesis of BKV T/t antigen, nor of HBV X protein, contrary to the original constructs) and expressed in COS-7 cells. These findings indicate that cell or vector-born factors are unlikely to be responsible for the aforementioned discrepancy. Another possibility is that, in earlier COS-7 experiments, the abundantly coexpressed M + S polypeptides could exert a helper effect, driving the secretion of L variants including the retention determinant identified here, thus masking its presence. Indeed, as shown by the cotransfection experiment reported here (Fig. 4), in the BKV-293 system M + S promote the export of a mutant (Δ2-19) bearing the C-terminal PreS1 determinant at high M + S to Δ2-19 ratios, while being inhibited in their own secretion at lower

ratios. Therefore, the M + S helper effect could account for the discrepancy with respect to secretion. However, a careful comparison of equivalent ayw and adw variants will be necessary in order to confirm the presence of the C-terminal PreS1 retention signal in the latter subtype (a nonconservative change is seen in adw amino acids corresponding to ayw 70–99—S88-T89-I90 in adw for P77-A78-N79 in ayw) and to check the activity of the previously identified adw N-terminal retention signal (aa 6–13) in the 293-BKV system.

The evidence for a PreS1 C-terminal portion crucial (at least in ayw L) for the HBsAg budding secretion inhibition raises questions on the nature of this stretch. Its function apparently requires that it is not prematurely sequestered in a canonical ER translocation pore (Gilmore, 1993), as happens by appending a dominant signal sequence ahead to PreS1 + 2. Possibly the critical stretch directly contacts, while at the cytosolic face of ER, other portions of the L molecule, imposing an overall folding incompatible with both precocious PreS1 + 2 translocation and subsequent HBsAg budding. Alternatively, the PreS1 C-terminal determinant could act as a signal diverting the protein to a distinct folding pathway by interaction with cell proteins. Running the 70–99 sequence against protein data banks did not reveal any strong homology with known proteins other than mammalian and avian *hepadnaviridae* L proteins. However, it is worth noting the presence of two proline-rich stretches (PANPPP, 78–83; PTPLSPP, 94–100) and the strict conservation of some of these residues in mammalian *hepadnaviridae.* Proline-rich stretches have been known as partners of SH3 domains (Ren *et al.,* 1993) and of peptidyl prolyl *cis–trans* isomerases (Kara Franke *et al.,* 1994).

A final possibility is that the PreS1 determinant is a target for modifications. In fact, Anderson and co-workers (Grgacic and Anderson, 1994) have recently demonstrated that duck hepatitis B virus (DHBV) L protein is serine/threonine phosphorylated within the PreS domain and that phosphorylation is conserved in virion envelopes, correlating with PreS domains buried in the interior of virions. The authors then speculate that DHBV PreS dephosphorylation is a requisite for its posttranslational translocation and further hypothesize that putative target sites (S/T-P sites) are similarly phosphorylated in HBV PreS1. However, no such modification has been described in mammalian *hepadnaviridae* L proteins. Consistently, in *in vivo* labeling experiments we failed to demonstrate any L phosphorylation in our system (data not shown). This is in keeping with the limited homology between mammalian and avian hepadnavirus Env proteins and their inability to complement one another in phenotypic mixing experiments (Gerhardt and Bruss, 1995).

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